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<p>(54) Title: NOVEL ANTIANGIOGENIC PEPTIDES</p> <p>(57) Abstract</p> <p>The present invention provides an antiangiogenic polypeptide having the amino acid sequence set forth in SEQ ID NO: 1 or a portion thereof which is effective to inhibit endothelial cell proliferation as determined by the capillary EC proliferation assay. Preferably, the portion has at least 50 % inhibition of bFGF-stimulated EC proliferation at 5 µg/ml, more preferably 75 % inhibition, most preferably 95 % inhibition.</p>		

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## NOVEL ANTIANGIOGENIC PEPTIDES

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

The present invention provides for novel antiangiogenic polypeptides and method of use thereof for treatment of diseases or disorders involving abnormal angiogenesis and tissue remodeling-associated conditions.

#### 2. Background

Blood vessels are the means by which oxygen and nutrients are supplied to living tissues and waste products are removed from living tissue. Angiogenesis refers to the process by which new blood vessels are formed. See, for example, the review by Folkman and Shing, *J. Biol. Chem.* 267, 10931-10934 (1992). Thus, where appropriate, angiogenesis is a critical biological process. It is essential in reproduction, development and wound repair. However, inappropriate angiogenesis can have severe negative consequences. For example, it is only after many solid tumors are vascularized as a result of angiogenesis that the tumors have a sufficient supply of oxygen and nutrients that permit it to grow rapidly and metastasize. Because maintaining the rate of angiogenesis in its proper equilibrium is so critical to a range of functions, it must be carefully regulated in order to maintain health. The angiogenesis process is believed to begin with the degradation of the basement membrane by proteolytic enzymes, e.g., metalloproteinases (MMPs) and plasminogen activator (PA), secreted from endothelial cells (EC) activated by mitogens such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).

Proteolytic activity is also required for the migration of EC into the perivascular stroma. These events are followed by sprout extension and subsequent lumen formation (Ausprunk, D.H., et al., *Microvascular Res.* 14:53-65 (1977)). As is EC "escape" from the parent venule, capillary sprout elongation, lumen formation, and EC migration are all events which are dependent on a shift in the proteolytic balance in favor of enzymatic activity (Ausprunk, D.H., et al., *Microvascular Res.* 14:53-65 (1977), Kalebic, T., et al. *Science*, 221:281-283 (1983), and (Moses, M.A., et al., *Science* 248:1408-1410 (1990)). Vascular morphogenesis

and invasion are also regulated by shifts in the finely tuned balance between proteases and their inhibitors (Liotta, L.A., et al., *Cell* 64:327-336, (1991); Moses, M.A., et al., *J. Cell Biochem.* 47:1-8 (1991); Herron, G. S., et al., *J. Biol. Chem.* 261:2814-2828 (1986), and Montesano, R., et. al., *Cell* 62:435-445, (1990)).

An accumulating body of evidence suggests that the remodeling of ECM that occurs during normal growth, wound repair and angiogenesis as well as during the development and progression of pathologic conditions including malignant diseases, is accomplished largely through the action of MMPs (Birkedal-Hansen, H. *Cell. Bio.* 7:728-735 (1985), Matrisian, L. *Trends Genet.* 6:121-125, (1990), and Woessner, J.F. *Acad. Sci.* 732:11-21 (1994), and Woessner, J.F. *Ann. N.Y. Acad. Sci.* 732:11-21, 1994.

The MMPs are members of a multigene family of metal-dependent enzymes. These proteases have been classified into four broad categories originally based on substrate specificity. These specific enzymes are the collagenases (MMP-1/EC3.4.24.7; MMP-8/EC3.4.24.34; MMP-13), the gelatinases A (MMP-2/EC3.4.24.24) and B (MMP-9/EC3.4.24.35), the stromelysins (MMP-3/EC3.4.24.17; MMP-10/EC3.4.24.22; MMP-1/EC3.4.24.7) including a metalloelastase (MMP-12), the membrane MMPs (MMP-14) (Birkedal-Hansen, H. *Current Opinions in Cell Biol.* 7:728-735, 1995. Matrisian, L. *Trends Genet.* 6:121-125, 1990. Woessner, J.F. *Ann. N.Y. Acad. Sci.* 732:11-21, 1994) and the family of membrane type MMPs (MT-MMP 1-4).

The regulation of MMP activity occurs at several levels including gene transcriptional control, proenzyme activation and inhibition of activated MMPs by endogenous inhibitors. Like many other enzyme families, the MMPs are a key component of a system of "balanced proteolysis" wherein a finely tuned equilibrium exists between the amount of active enzyme and its proteinase inhibitor(s) (Liotta, L.A., et al., *Cell* 64:327-336, (1991)). These native metalloproteinase inhibitors comprise a family of proteins generally referred to as the TIMPS (Tissue Inhibitor of MetalloProteinase) (Docherty, A.J.P., et al., *Nature* 318:66-69, (1985), Carmichael, D.F., et al. *Proc. Natl. Acad. Sci. USA* 83:2407-2411, (1986); Moses, M.A., et al., *J. Cell. Biochem.* 47:230-235, (1991); Murray, J.B., et al., *J. Biol. Chem.* 261:4154-4159 (1986); Stetler-Stevenson, W.G., et al., *J. Biol. Chem.* 269:17374-17378, (1989); Pavloff, N., et al., *J. Biol. Chem.* 267:17321-17326, (1992), and DeClerck, T.A., et al., *J. Biol. Chem.* 264:17445-17453 (1989)). They bind to

activate MMPs with 1:1 molar stoichiometry. There are also a number of less well-characterized lower molecular weight metalloproteinase inhibitors which await complete purification and identification.

The TIMPs consist of six disulfide-bonded loops. Deletion mutagenesis studies have demonstrated that two structurally distinct domains can be defined, the N-terminal domain consisting of loops 1-3 and the C-terminal domain consisting of loops 4-6 (Murphy, G. Houbrechts., et al. *Biochemistry* 30(33):8097-8101, (1991); Willenbrock, F., et al., *Biochem.* 32:4330-4337, (1993), and Nguyen, Q., et al., *Biochem.* 33:2089-2095, (1994)).

Much research attention has been focused on studies aimed at defining the domains of TIMPs that are important to their ability to inhibit MMP activity. Construction of truncated forms of these molecules has provided some insight. Residues 1-126 of TIMP-1 and 1-127 of TIMP-2 which contain three of the six disulfide bonds in the full-length molecules have been expressed in mammalian cells in the absence of the C-terminal region and are secreted in a soluble form (Murphy, G., et al. *Biochemistry* 30(33):8097-8101, (1991)). These truncated forms inhibit matrilysin and the catalytic domains of stromelysin and gelatinase A, demonstrating that there is a direct interaction between the N-terminal domain of the TIMPs and the catalytic domains of the MMPs (Murphy, G., et al. *Biochemistry* 30(33):8097-8101, (1991); Willenbrock, F., et al., *Biochem.* 32:4330-4337, (1993), and Nguyen, Q., et al., *Biochem.* 33:2089-2095, (1994)). The structure of the N-terminal domain of either TIMP-1 or TIMP-2 is not affected by the C-terminal domain (Nguyen, Q., et al., *Biochemistry* 33:2089-2095, (1994)).

A significant number of mutational analyses also support the concept that the NH<sub>2</sub>-terminal domain of TIMP-1 (Cys<sup>1</sup>-Glu<sup>126</sup>) is sufficient for inhibition of MMPs (Wilhelm, S.M., et al., *J. Biol. Chem.* 264:17213-17221, (1989), Murphy, G., et al., *Biochemistry* 30(33):8097-8101, (1991); Murphy, G., et al., *Bio. Chem. Biophys. Acta.* 839:214-218, (1985); Stricklin, G.P., *Collagen Relat. Res.* 6:219-228, (1986); Tolley, S.P., et al., *Protein: Struct., Fuct., Genet.* 17:435-437, (1993), and Howard, E.W., et al., *J. Biol. Chem.* 266:13064-13069, (1991)). Furthermore, single-residue mutations in the region bounded by Cys3 and Cys13 caused an increase of 8-fold in K<sub>when</sub> compared with wild type TIMP-1 (O'Shea, M., et al., *Biochemistry* 31(42):10146-10151, (1992)). A series of experiments including competition studies with synthetic peptides and localization of epitopes of blocking

antibodies revealed that the region marking the transition between the NH<sub>2</sub>-terminal and COOH-terminal domains of the TIMP-1 molecule may be particularly important for its ability to inhibit collagenase (Bodden, M.K., et al., *J. Biol. Chem.* 269:18943-18952, (1994)).

It is now widely accepted that the N-terminal domain of the TIMPs represent a stable, minimized form of the inhibitor that includes the major site or sites necessary for MMP inhibition (Williamson, R.A., et al., *Biochem.* 33:11745-11759, (1994)). Site-directed mutagenesis studies on TIMP-1 have demonstrated that no single residue is likely to be responsible for MMP inhibition (O'Shea, M., et al., *Biochem.* 10146-10151, (1992)).

The C-terminal domain of TIMPs also makes some binding contribution to the TIMP-MMP complex, in particular, the C-terminal domain of TIMP-2 which may be responsible for the specific interaction of this molecule with progelatinase A (Willenbrock, F., et al., *Biochemistry* 32:4330-4337, (1993)). Mutational studies also support the idea that the COOH-terminal domain of TIMP-2 which does not appear to be required for MMP inhibition (O'Shea, M., et al., *Biochemistry* 31(42):10146-10151 (1992)) interacts with the pexin-like domain of gelatinase A (Hayakawa, T., et al., *FEBS Lett.* 298:29-32, (1992)). This interaction has been shown to prevent autodegradation of the enzyme (Goldberg, G.I., et al., *J. Biol. Chem.* 267:4583-4591 (1992), Bodden, M.K., et al., *Biol. Chem.* 269:18943-18952, (1994), and Howard, E.W., et al., *J. Biol. Chem.* 266:13084-13089, (1991)). The C-terminal domain of both TIMP-1 and TIMP-2 participate in low-affinity interactions with the C-terminal domain of gelatinase A which increases the rate of association by a factor of about 100 in both cases (Willenbrock, F., et al., *Biochemistry* 32:4330-4337, (1993)).

TIMPs have been shown to inhibit the migration of endothelial cells *in vitro* and, depending on the model used, angiogenesis *in vivo*. (Mignatti, et al. *J. Cell Bio.* 108:671-682, (1989)) first demonstrated that TIMP-1 could inhibit migration of microvascular cells *in vitro* using the amnion invasion assay. Montesano and coworkers later showed that a collagenase inhibitor (1, 10-phenanthroline) could inhibit capillary tube formation *in vitro* (Montesano, R., et. al. *Cell* 42:489-477, (1985)). Since it was first demonstrated that a TIMP purified from a vascular cartilage was a potent inhibitor of angiogenesis *in vivo* and EC proliferation and migration *in vitro* (Moses, M.A., et al., *Science* 248:1408-1410, (1990); Moses, M.A.,

et al., *J. Cell Biol.* 119:475-482, (1992) and Murphy, A.N. et al., *J. Cell. Phys.* 157:351-358 (1993)) showed that TIMP-2, but not TIMP-1, inhibited FGF-stimulated endothelial cell proliferation. TIMP-1 has been shown to stimulate the growth of EC proliferation in other studies as well (Hayakawa, T., et al *FEBS Letts.* 298:29-32, (1992)). TIMPs have been shown to inhibit neovascularization in various *in vivo* models (Takigawa, M., et al., *Biochem. Biophys. Res. Commun.* 171:1264-1271 and Johnson, M.D. et al., *J. Cell. Physiol.* 160:194-202, (1989)).

Much research attention has focused on studies aimed at defining the domain of TIMPs that are important to their ability to inhibit MMP activity. It is now widely accepted that the N-terminal domain of the TIMPs represents a stable, minimized form of the inhibitor that includes the major site or sites necessary for MMP inhibition (Williamson, R.A. et al. *Biochemistry* 33:11745-11759 (1994)). The C-terminal domain of TIMPs also makes some binding contribution to the TIMP-MMP complex, in particular, the C-terminal domain of TIMP-2 which may be responsible for the specific interaction of this molecule with progelatinase A (Willenbrock, F. et al., *Biochemistry* 32:4330-4337 (1993)).

It has been suggested that the regions of amino acid sequences between TIMP-1 and TIMP-2 that are highly conserved such as the N-terminus, may be responsible for the known shared functions of these proteins, for example, inhibition of activated MMPs and their shared ability to inhibit FGF-stimulated capillary EC migration (Moses, M.A. et al., *Science* 248:1408-1410 (1990); Moses, M.A. et al., *J. Cell Biol.* 119:475-482 (1992); Mignatti, P. et al., *J. Cell Bio.* 108:671-682 (1989) and Murphy, A.N. et al., *J. Cell. Phys.* 157:351-358 (1993)). Areas of low homology, for example, the C-terminus, may be responsible for those functions which are unique for the individual TIMPs (Stetler-Stevenson et al., *J. Biol. Chem.* 265(23):13933-13936 (1990)). These include the binding of TIMP-2 to the latent form of gelatinase A and the failure of TIMP-2 antibodies to detect TIMP-1 (Stetler-Stevenson, W.G. et al., *J. Biol. Chem.* 265(23):13933-13936 (1990)).

The rate of angiogenesis involves a change in the local equilibrium between positive and negative regulators of the growth of microvessels. The therapeutic implications of angiogenic growth factors were first described by Folkman and colleagues over two decades ago (Folkman, *N. Engl. J. Med.*, 285:1182-1186 (1971)). Abnormal angiogenesis occurs when the body loses at least some control of angiogenesis, resulting in either excessive or insufficient blood vessel growth. For

instance, conditions such as ulcers, strokes, and heart attacks may result from the absence of angiogenesis normally required for natural healing. In contrast, excessive blood vessel proliferation can result in tumor growth, tumor spread, blindness, psoriasis and rheumatoid arthritis.

Thus, there are instances where a greater degree of angiogenesis is desirable-- increasing blood circulation, wound healing, and ulcer healing. For example, recent investigations have established the feasibility of using recombinant angiogenic growth factors, such as fibroblast growth factor (FGF) family (Yanagisawa-Miwa, et al., *Science*, 257:1401-1403 (1992) and Baffour, et al., *J Vasc Surg*, 16:181-91 (1992)), endothelial cell growth factor (ECGF)(Pu, et al., *J Surg Res*, 54:575-83 (1993)), and more recently, vascular endothelial growth factor (VEGF) to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia (Takeshita, et al., *Circulation*, 90:228-234 (1994) and Takeshita, et al., *J Clin Invest*, 93:662-70 (1994)).

Conversely, there are instances, where inhibition of angiogenesis is desirable. For example, many diseases are driven by persistent unregulated angiogenesis, also sometimes referred to as "neovascularization." In arthritis, new capillary blood vessels invade the joint and destroy cartilage. In diabetes, new capillaries invade the vitreous, bleed, and cause blindness. Ocular neovascularization is the most common cause of blindness. Tumor growth and metastasis are angiogenesis-dependent. A tumor must continuously stimulate the growth of new capillary blood vessels for the tumor itself to grow.

The current treatment of these diseases is inadequate. Agents which prevent continued angiogenesis, e.g, drugs (TNP-470), monoclonal antibodies, antisense nucleic acids and proteins (angiostatin and endostatin) are currently being tested. See, Battegay, *J. Mol. Med.*, 73, 333-346 (1995); Hanahan et al., *Cell*, 86, 353-364 (1996); Folkman, *N. Engl. J. Med.*, 333, 1757-1763 (1995). Although preliminary results with the antiangiogenic proteins are promising, they are relatively large in size and they are difficult to use and produce. Moreover, proteins are subject to enzymatic degradation. Thus, new agents that inhibit angiogenesis are needed. New antiangiogenic peptides that show improvement in size, ease of production, stability and/or potency would be desirable.



## SUMMARY OF THE INVENTION

We have discovered that surprisingly peptide fragments of the carboxy terminus of TIMP-2 comprising no more than 35% of the TIMP-2 protein, do not inhibit MMP activity but have endothelial cell inhibitory activity, i.e., the ability to inhibit endothelial cell migration and proliferation. Thus these fragments are useful as antiangiogenic agents.

The present invention provides an antiangiogenic polypeptide having the following amino acid sequence

CKITRCPMIPCYISSPDECLWMDWVTEKNINGHQAKFFACIKRSDGSCAWYRGA APPKQEFLDIEDP (SEQ ID NO: 1) or a portion thereof which is effective to inhibit endothelial cell proliferation as determined by the capillary EC proliferation assay. Preferably, the portion has at least 50% inhibition of bFGF-stimulated EC proliferation at 5ug/ml, more preferably 75% inhibition, most preferably 95% inhibition.

Preferred antiangiogenic polypeptides of the invention include:

KITRCPMIPC (SEQ ID NO: 2) (aa 129-138; partial loop-4 + loop-5);  
KITRCPMIPCYISSPDE (SEQ ID NO: 3) (aa 129-145; partial loop-4 + loop-5);  
CKITRCPMIPCYISSPDECLWMDWVTEKNINGHQAKFFAC (SEQ ID NO: 4) (aa 128-167; partial loop-4 + loop-5 + loop-6); CLWMDWVTEKNINGHQAKFFAC (SEQ ID NO: 5) (aa 146-167; loop-5);  
CKITRCPMIPCYISSPDECLWMDWVTEKNINGHQAKFFACIKRSDGSC (SEQ ID NO: 6) (AA 128-175; loop-4 + loop-5 + loop-6); and AWYRGAAPPKQEFLDIEDP (SEQ ID NO: 7) (aa 176-194).

The present invention further provides an antiangiogenic polypeptide comprising the amino acid sequence KITRCPMIPC (SEQ ID NO: 2), but excluding the full sequence of human TIMP-2 (SEQ ID NO:8, U.S. Patent 5,595,885).

The present invention also provides pharmaceutical compositions comprising antiangiogenic polypeptides or nucleic acids encoding such a polypeptide, in therapeutically effective amounts that are capable of inhibiting endothelial cell proliferation, and their methods of use.

The invention further provides treatment of neovascular disorders by administration of a pharmaceutical composition comprising an antiangiogenic polypeptide of the invention or nucleic acid encoding such a polypeptide, and a pharmaceutically acceptable carrier.

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The invention also provides for treatment of a class of disorders characterized as tissue remodeling-associated conditions, which include cancers, arthritic conditions, obstructive disorders, degenerative disorders, and problematic wound-healing and ulcerative disorders.

In one embodiment, a pharmaceutical composition of the invention is administered to treat a cancerous condition, or to prevent progression from the pre-neoplastic or pre-malignant state into a neoplastic or a malignant state. In other specific embodiments, a pharmaceutical composition of the invention is administered to treat ocular disorders associated with neovascularization.

Other aspects of the invention are disclosed *infra*.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an antiangiogenic polypeptide having the amino acid sequence set forth in SEQ ID NO: 1 or a portion thereof which is effective to inhibit endothelial cell proliferation as determined by the capillary EC proliferation assay. Preferably, the portion has at least 50% inhibition of bFGF-stimulated EC proliferation at 5 µg/ml, more preferably 75% inhibition, most preferably 95% inhibition.

Preferred antiangiogenic polypeptides of the invention include:

KITRCPMIPC (SEQ ID NO: 2) (aa 129-138; partial loop-4 + loop-5);  
KITRCPMIPCYISSPDE (SEQ ID NO: 3) (aa 129-145; partial loop-4 + loop-5);  
CKITRCPMIPCYISSPDECLWMDWVTEKKNINGHQAKFFAC (SEQ ID NO: 4) (aa 128-167; partial loop-4 + loop-5 + loop-6); CLWMDWVTEKKNINGHQAKFFAC (SEQ ID NO: 5) (aa 146-167; loop-5);  
CKITRCPMIPCYISSPDECLWMDWVTEKKNINGHQAKFFACIKRSDGSC (SEQ ID NO: 6) (AA 128-175; loop-4 + loop-5 + loop-6); and AWYRGAAPPKQEFLDIEDP (SEQ ID NO: 7) (aa 176-194).

The present invention further provides an antiangiogenic polypeptide comprising the amino acid sequence KITRCPMIPC (SEQ ID NO: 2), but excluding the full sequence of human TIMP-2 (SEQ ID NO:8, U.S. Patent 5,595,885).

Antiangiogenic polypeptides of the invention can be combined with a therapeutically effective amount of another molecule which negatively regulates angiogenesis which may be, but is not limited to, platelet factor 4, thrombospondin-1, tissue inhibitors of metalloproteases, prolactin (16-Kd

fragment), angiostatin (38-Kd fragment of plasminogen), endostatin, bFGF soluble receptor, transforming growth factor beta, interferon alfa, and placental proliferin-related protein.

An antiangiogenic polypeptide of the invention may also be combined with chemotherapeutic agents.

Analogous of the antiangiogenic polypeptides of the invention can be made by altering the protein sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. These include altering sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

The antiangiogenic polypeptides and analogs of the invention can be derived from tissue or produced by various methods known in the art. The manipulations, which result in their production, can occur at the gene or protein level. For example, a cloned gene sequence coding for an antiangiogenic polypeptide can be modified by any of numerous strategies known in the art. Sambrook et al., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog, care should be taken to ensure that the modified gene remains within the same translational reading frame as the troponin subunit gene, uninterrupted by translational stop signals, in the gene region where the desired troponin activity is encoded.

The antiangiogenic polypeptides are preferably produced by recombinant methods, including, for example, the yeast expression system *Pichia pastoris*. See the procedures disclosed in Example 1, which follows.

The term "isolated" means that the polypeptide is removed from its original environment. For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or DNA or polypeptides, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

Where it is desired to express a polypeptide of the invention any suitable system can be used. The general nature of suitable vectors, expression vectors and constructions therefor will be apparent to those skilled in the art.

Suitable expression vectors may be based on phages or plasmids, both of which are generally host-specific, although these can often be engineered for other hosts. Other suitable vectors include cosmids and retroviruses, and any other vehicles, which may or may not be specific for a given system. Control sequences, such as recognition, promoter, operator, inducer, terminator and other sequences essential and/or useful in the regulation of expression, will be readily apparent to those skilled in the art.

Correct preparation of nucleotide sequences may be confirmed, for example, by the method of Sanger et al. (*Proc. Natl. Acad. Sci. USA* 74:5463-7 (1977)).

A DNA fragment encoding an antiangiogenic polypeptide may readily be inserted into a suitable vector. Ideally, the receiving vector has suitable restriction sites for ease of insertion, but blunt-end ligation, for example, may also be used, although this may lead to uncertainty over reading frame and direction of insertion. In such an instance, it is a matter of course to test transformants for expression, 1 in 6 of which should have the correct reading frame. Suitable vectors may be selected as a matter of course by those skilled in the art according to the expression system desired.

By transforming a suitable organism or, preferably, eukaryotic cell line, such as HeLa, with the plasmid obtained, selecting the transformant with ampicillin or by other suitable means if required, and adding tryptophan or other suitable promoter-inducer (such as indoleacrylic acid) if necessary, the desired polypeptide

or protein may be expressed. The extent of expression may be analyzed by SDS polyacrylamide gel electrophoresis-SDS-PAGE (Lemelli, *Nature* 227:680-685 (1970)).

Suitable methods for growing and transforming cultures etc. are usefully illustrated in, for example, *Molecular Cloning, A Laboratory Manual* (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor), *Current Protocols in Molecular Biology* (Eds. Ausubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, N.Y. 1992).

The polypeptides and proteins may be isolated from the fermentation or cell culture and purified using any of a variety of conventional methods including: liquid chromatography such as normal or reversed phase, using HPLC, FPLC and the like; affinity chromatography (such as with inorganic ligands or antibodies); size exclusion chromatography; immobilized metal chelate chromatography; gel electrophoresis; and the like. One of skill in the art may select the most appropriate isolation and purification techniques without departing from the scope of this invention.

The polypeptides may be generated by any of several chemical techniques. For example, they may be prepared using the solid-phase synthetic technique originally described by R. B. Merrifield, "Solid Phase Peptide Synthesis. I. The Synthesis Of A Tetrapeptide", *J. Am. Chem. Soc.*, 83, pp. 2149-54 (1963), or they may be prepared by synthesis in solution. A summary of peptide synthesis techniques may be found in E. Gross & H. J. Meinhofer, *The Peptides: Analysis, Synthesis, Biology; Modern Techniques Of Peptide And Amino Acid Analysis*, John Wiley & Sons, (1981) and M. Bodanszky, *Principles Of Peptide Synthesis*, Springer-Verlag (1984).

The functional activity and/or therapeutically effective dose of an antiangiogenic polypeptide or nucleic acid encoding therefor can be assayed in vitro by various methods. For example, where one is assaying for the ability of the angiogenic inhibitory polypeptides, fragments, and analogs, to inhibit or interfere with the proliferation of capillary endothelial cells (EC) in vitro, various bioassays known in the art can be used, including, but not limited to, radioactive incorporation into nucleic acids, calorimetric assays and cell counting.

Inhibition of endothelial cell proliferation may be measured by calorimetric determination of cellular acid phosphatase activity or electronic cell counting.

These methods provide a quick and sensitive screen for determining the number of endothelial cells in culture after treatment with the connective tissue growth factor protein, derivative, or analog of the invention, and an angiogenesis stimulating factor such as aFGF. The calorimetric determination of cellular acid phosphatase activity is described by Connolly et al., 1986, *J. Anal. Biochem.* J52: 136-140. According to this method, capillary endothelial cells are treated with angiogenesis stimulating factors, such as aFGF, and a range of potential inhibitor concentrations. These samples are incubated to allow for growth, and then harvested, washed, lysed in a buffer containing a phosphatase substrate, and then incubated a second time. A basic solution is added to stop the reaction and color development is determined at 405 $\lambda$ . According to Connolly et al., a linear relationship is obtained between acid phosphatase activity and endothelial cell number up to 10,000 cells/sample. Standard curves for acid phosphatase activity are also generated from known cell numbers in order to confirm that the enzyme levels reflect the actual EC numbers. Percent inhibition is determined by comparing the cell number of samples exposed to stimulus with those exposed to both stimulus and inhibitor.

The incorporation of radioactive thymidine by capillary endothelial cells represents another means by which to assay for the inhibition of endothelial cell proliferation by a potential angiogenesis inhibitor. According to this method, a predetermined number of capillary endothelial cells are grown in the presence of 3H-Thymidine stock, an angiogenesis stimulator such as for example, bFGF, and a range of concentrations of the angiogenesis inhibitor to be tested. Following incubation, the cells are harvested and the extent of thymidine incorporation is determined.

The ability of varying concentrations of antiangiogenic polypeptides to interfere with the process of capillary endothelial cell migration in response to an angiogenic stimulus can be assayed using the modified Boyden chamber technique.

Another means by which to assay the functional activity of antiangiogenic polypeptides involves examining the ability of the compounds to inhibit the directed migration of capillary endothelial cells which ultimately results in capillary tube formation. This ability may be assessed for example, using an assay in which capillary endothelial cells plated on collagen gels are challenged with the inhibitor,

and determining whether capillary-like tube structures are formed by the cultured endothelial cells.

Assays for the ability to inhibit angiogenesis *in vivo* include the chick chorioallantoic membrane assay and mouse, rat or rabbit corneal pocket assays. See, Polverini et al., 1991, *Methods Enzymol.* 198: 440-450. In corneal pocket assays, a tumor of choice is implanted into the cornea of the test animal in the form of a corneal pocket. The potential angiogenesis inhibitor is applied to the corneal pocket and the corneal pocket is routinely examined for neovascularization.

The ability of the antiangiogenic polypeptides to influence angiogenesis can also be determined using a number of known *in vivo* and *in vitro* assays. Such assays are disclosed in Jain et al., *Nature Medicine* 3, 1203-1208(1997), the disclosure of which is herein incorporated by reference.

The therapeutically effective dosage for inhibition of angiogenesis *in vivo*, defined as inhibition of capillary endothelial cell proliferation, migration, and/or blood vessel growth, may be extrapolated from *in vitro* inhibition assays using the compositions of the invention above or in combination with other angiogenesis inhibiting factors. The effective dosage is also dependent on the method and means of delivery. For example, in some applications, as in the treatment of psoriasis or diabetic retinopathy, the inhibitor is delivered in a topical-ophthalmic carrier. In other applications, as in the treatment of solid tumors, the inhibitor is delivered by means of a biodegradable, polymeric implant. The protein can also be modified, for example, by polyethyleneglycol treatment.

Diseases, disorders, or conditions, associated with abnormal angiogenesis or neovascularization, and can be treated with a therapeutic compound of the invention include, but are not limited to retinal neovascularization, tumor growth, hemangioma, solid tumors, leukemia, metastasis, psoriasis, neovascular glaucoma, diabetic retinopathy, arthritis, endometriosis, and retinopathy of prematurity (ROP).

The term "effective amount" refers to an amount of the antiangiogenic polypeptide of the invention sufficient to exhibit a detectable therapeutic effect. The therapeutic effect may include, for example, without limitation, inhibiting the growth of undesired tissue or malignant cells, inhibiting inappropriate angiogenesis (neovascularization), limiting tissue damage caused by chronic inflammation, inhibition of tumor cell growth, and the like. The precise effective amount for a

subject will depend upon the subject's size and health, the nature and severity of the condition to be treated, and the like. Thus, it is not possible to specify an exact effective amount in advance. However, the effective amount for a given situation can be determined by routine experimentation based on the information provided herein.

The term "pharmaceutically acceptable" refers to compounds and compositions which may be administered to mammals without undue toxicity. Exemplary pharmaceutically acceptable salts include mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like.

The antiangiogenic polypeptides of the invention are administered orally, topically, or by parenteral means, including subcutaneous and intramuscular injection, implantation of sustained release depots, intravenous injection, intranasal administration, and the like. Accordingly, antiangiogenic polypeptides of the invention are preferably administered as a pharmaceutical composition comprising an antiangiogenic polypeptides of the invention in combination with a pharmaceutically acceptable carrier. Such compositions may be aqueous solutions, emulsions, creams, ointments, suspensions, gels, liposomal suspensions, and the like. Suitable carriers (excipients) include water, saline, Ringer's solution, dextrose solution, and solutions of ethanol, glucose, sucrose, dextran, mannose, mannitol, sorbitol, polyethylene glycol (PEG), phosphate, acetate, gelatin, collagen, Carbopol Registered TM, vegetable oils, and the like. One may additionally include suitable preservatives, stabilizers, antioxidants, antimicrobials, and buffering agents, for example, BHA, BHT, citric acid, ascorbic acid, tetracycline, and the like. Cream or ointment bases useful in formulation include lanolin, Silvadene Registered TM (Marion), Aquaphor Registered TM (Duke Laboratories), and the like. Other topical formulations include aerosols, bandages, and other wound dressings. Alternatively one may incorporate or encapsulate the therapeutic compound of the invention in a suitable polymer matrix or membrane, thus providing a sustained-release delivery device suitable for implantation near the site to be treated locally. Other devices include indwelling catheters and devices such as the Alzet Registered TM minipump. Ophthalmic preparations may be formulated using commercially available vehicles such as Sorbi-care Registered TM (Allergan), Neodecadron Registered TM (Merck, Sharp & Dohme), Lacrilube Registered TM, and the like, or



may employ topical preparations such as that described in U.S. Pat. No. 5,124,155, incorporated herein by reference. Further, one may provide a therapeutic compound of the invention in solid form, especially as a lyophilized powder. Lyophilized formulations typically contain stabilizing and bulking agents, for example human serum albumin, sucrose, mannitol, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co.).

The DNA encoding antiangiogenic polypeptides of the invention can be used in the form of gene therapy and delivered to a host by any method known to those of skill in the art to treat disorders associated with angiogenic or tissue remodeling-associated conditions.

A preferred embodiment of the present invention relates to methods of inhibiting angiogenesis of solid tumors to prevent further tumor growth and eventual metastasis. To this end, any solid tumor or the region surrounding the tumor accessible to gene transfer will be a target for the disclosed therapeutic applications. A DNA encoding an angiogenic polypeptide, housed within a recombinant viral- or non-viral-based gene transfer system may be directed to target cells within proximity of the tumor by any number of procedures known in the art, including but not limited to (a) surgical procedures coupled with administration of an effective amount of the DNA to the site in and around the tumor (involving initial removal of a portion or the entire tumor, if possible); (b) injection of the gene transfer vehicle directly into or adjacent to the site of the tumor; and, (c) localized or systemic delivery of the gene transfer vector and/or gene product using techniques known in the art.

Any solid tumor that contains angiogenic protein expressing cells will be a potential target for treatment. Examples, but by no means listed as a limitation, of solid tumors which will be particularly vulnerable to gene therapy applications are (a) neoplasms of the central nervous system such as, but again not necessarily limited to glioblastomas, astrocytomas, neuroblastomas, meningiomas, ependymomas; (b) cancers of hormone-dependent, tissues such as prostate, testicles, uterus, cervix, ovary, mammary carcinomas including but not limited to carcinoma in situ, medullary carcinoma, tubular carcinoma, invasive (infiltrating) carcinomas and mucinous carcinomas; (c) melanomas, including but not limited to cutaneous and ocular melanomas; (d) cancers of the lung which at least include

squamous cell carcinoma, spindle carcinoma, small cell carcinoma, adenocarcinoma and large cell carcinoma; and (e) cancers of the gastrointestinal system such as esophageal, stomach, small intestine, colon, colorectal, rectal and anal region which at least include adenocarcinomas of the large bowel.

A DNA fragment encoding an antiangiogenic polypeptide may be delivered either systemically or to target cells in the proximity of a solid tumor of the mammalian host by viral or non-viral based methods. Viral vector systems which may be utilized in the present invention include, but are not limited to, (a) adenovirus vectors; (b) retrovirus vectors; (c) adeno- associated virus vectors; (d) herpes simplex virus vectors; (e) SV 40 vectors; (f) polyoma virus vectors; (g) papilloma virus vectors; (h) picornavirus vectors; and (i) vaccinia virus vectors.

The recombinant virus or vector containing the DNA encoding the antiangiogenic polypeptide of the present invention is preferably administered to the host by direct injection into a solid tumor and/or quiescent tissue proximal to the solid tumor, such as adipose or muscle tissue. It will of course be useful to transfect tumor cells in the region of targeted adipose and muscle tissue. Transient expression of the antiangiogenic polypeptide in these surrounding cells will result in a local extracellular increase in these proteins.

Non-viral vectors which are also suitable include DNA-lipid complexes, for example liposome-mediated or ligand/ poly-L-Lysine conjugates, such as asialoglyco-protein-mediated delivery systems (see, e.g., Felgner et al., 1994, J. Biol. Chem. 269: 2550-2561; Derossi et al., 1995, Restor. Neurol. Neuros. 8: 7-10; and Abcallah et al., 1995, Biol. Cell 85:1-7). Direct injection of "naked" DNA may also be used.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

The references cited throughout this application are herein incorporated by reference.

The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

## EXAMPLES

### Cloning and Expression of TIMP Domains

The yeast expression system *Pichia pastoris* (Invitrogen) was used to express full length TIMPs as well as various domains of these inhibitors. *Pichia pastoris* yeast expression has been successfully utilized to produce large quantities of disulfide-bonded proteins (White, C. E., et al. *Structure* 2:1003-1005, (1994)). This is of particular importance given the fact that the TIMPs contain 6 disulfide bonds which are highly conserved. This expression system has a number of significant advantages over others. First, proteins produced in *P. pastoris* are usually folded correctly because yeast provides an intracellular folding environment similar to that of mammalian cells. A number of disulfide-bonded proteins have now been successfully expressed in *P. pastoris* including epidermal growth factor, EGF (Clare, J. J., et al. *Gene* 105:205-212, (1991)), aprotinin (Vedvick, T., et al., *Indust. Microbiol.* 7:199-202, (1991)) as well as proteins requiring mammalian post-translational modifications, such as tumor necrosis factor (Sreekrishna, K., et al., *Biotechnology* 28:4117-4125, (1989)), invertase (Tschopp, J.J., et al., *Biotechnology* 5:1305-1308, (1987)) and others. Second, secretion of proteins into the medium surrounding *P. pastoris* yeast cells can be easily accomplished, providing a substantial improvement in purification over other methods. Third, high yields of protein per liter of culture (typically 100-500 mg/L) can be obtained from multicopy inserts when expression is carried out in a fermenter. Fourth, transformation and growth of *P. pastoris* does not require special hoods, incubators or techniques other than those used for *E. coli*. Finally, spheroblast transformation produced multicopy insertion of the gene of interest, vastly increasing the yield of protein and it is easy to select for multicopy insert transformants (White, C.E., et. al., *Structure* 2:1003-1005, (1994)).

We successfully expressed full length human TIMP-3 in the *P. pastoris* system. Cloning vector PCR II carrying the full-length human TIMP-3 gene was subjected to digestion with restriction enzymes EcoR I and Not I to release the fragment of cDNA containing TIMP-3. Following gel extraction of the fragment,

ligation was performed with the *Pichia* shuttle vector pPIC9K (Invitrogen) digested with EcoR I and Not I. Aliquots of the ligation mixture were used to transform competent *E. coli* XL2-Blue. Minipreps of the resulting clones were analyzed by restriction analysis using EcoR I and Not I. A construct having the correct orientation was designated TIMP3-pPICK. This recombinant plasmid was then linearized with Bgl II and transformed by electroporation in *Pichia* GS115-competent cells, resulting in recombination of the plasmid at the AOX1 site of the genome to encode a nonfusion protein. The *his4* GS115 should now be *HIS4* Mut<sup>s</sup>, and can be selected for a plating in histidine deficient plates followed by screening for retarded growth in methanol plates.

After 24 hours, positive colonies were harvested, pooled and re-plated into minimal plates. Since multiple copies of the construct are reported to confer resistance to high levels of GF18 we screened for "jackpot" clones by replica plating in increasing doses of GF18, as high as 4 mg/ml. After four days, approximately five colonies containing multiple copies of the gene were screened for expression levels of TIMP-3 in 5ml cultures. The two clones showing the highest expression, T3-3 His+Mut<sup>s</sup> and T3-A His+Mut<sup>s</sup>, were then used to inoculate 500 ml cultures, as was one clone containing pPIC9K vector alone as a control for background expression. Both T3-3 and T3-A produced a prominent protein band at app. 25 kDa by silver-stained SDS-PAGE, while vector control showed no band at the same molecular weight. Highly purified TIMP-3 was obtained by subsequent chromatography on a Progel-TSK G30000SWK HPLC column (Supelco) followed by SDS-PAGE and silver staining. Immunoblot analysis using TIMP-3 specific antibodies (Triple Point Biologics) demonstrated cross-reactivity of the 25 kDa band. Both T3-3 and T3-A were inhibitory when tested in the standard radiometric enzyme assay for MMP inhibition (Moses, M.A., et al., *Science* 248:1408-1410, (1990) and Moses, M.A., et al., *J. Cell Biol.* 119:475-482, (1992)).

We have followed the protocols described above for the expression of TIMP-3 with the exception being the cloning vector PCR II carried the C-terminal domain of human TIMP-2 consisting of loop 4-6 (Murphy, G., et al., *Biochemistry* 30(33):8089-8101, (1991); Willenbrock, F., et al., *Biochemistry* 32:4330-4337, (1993) and Nguyen, G., et al., *Biochemistry* 33:2089-2095, (1994)). The transformed colonies were screened for G418 resistance and one colony was found to be resistant to 1 mg/ml G418. This clone, T2C-11.1 was screened for expression levels along with five

colonies resistant to various lower levels of G418. All clones were analyzed by SDS-PAGE followed by silver staining and a band at approximately 7,800-8,000 Da was detecting for each sample. Of all, T2C-11.1 produced the highest yield of expressed protein. T2C-11.1 was then used to inoculate 500 ml cultures, as was a clone transformed with vector alone for control. We purified the 7,800 Da protein using standard chromatographic strategies.

The purified protein inhibited bFGF-stimulated EC proliferation in the bFGF stimulated EC proliferation assay (Moses, et al., *Science*, 248:1408-1410 (1990)) (Figure 1) and inhibited neovascularization *in vivo* in the CAM assay. The purified protein did not inhibit MMP-1 activity (Figure 2).

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

-20-

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/ISA00/07156

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING** This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-5, drawn to an antiangiogenic polypeptide, a pharmaceutical composition and a method of treating neurovascular disorder.

Group II, claim(s) 6, drawn to a method of treating tissue remodeling.

Group III, claim(s) 7-9, drawn to a method of treating atopic angiogenesis in a subject.

Group IV, claim(s) 10, drawn to a method of treating ophthalmologic disorder.

Group V, claim(s) 11-13, drawn to polynucleotide sequences, a vector and a host cell containing the vector.

The inventions listed as Groups I-V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature linking groups I-V appears to be that they relate to an antiangiogenic polypeptide.

However, Takigawa et al. (1990) teach that metalloproteinases are involved in angiogenesis and this can be inhibited by polypeptides which are termed tissue inhibitor metalloproteinases (TIMP). Therefore, the technical feature linking the inventions of groups I-V does not constitute a special technical feature as defined by PCT rule 13.2, as it does not define a contribution over the prior art.

The special technical feature of group I is considered to be the polypeptide and a method of treating neurovascular disorder.

The special technical feature of group II is considered to be a method of treating tissue remodeling.

The special technical feature of group III is considered to be a method of inhibiting atopic angiogenesis.

The special technical feature of group IV is considered to be treating ophthalmologic disorder.

The special technical feature of group V is considered to be a polynucleotide sequence.

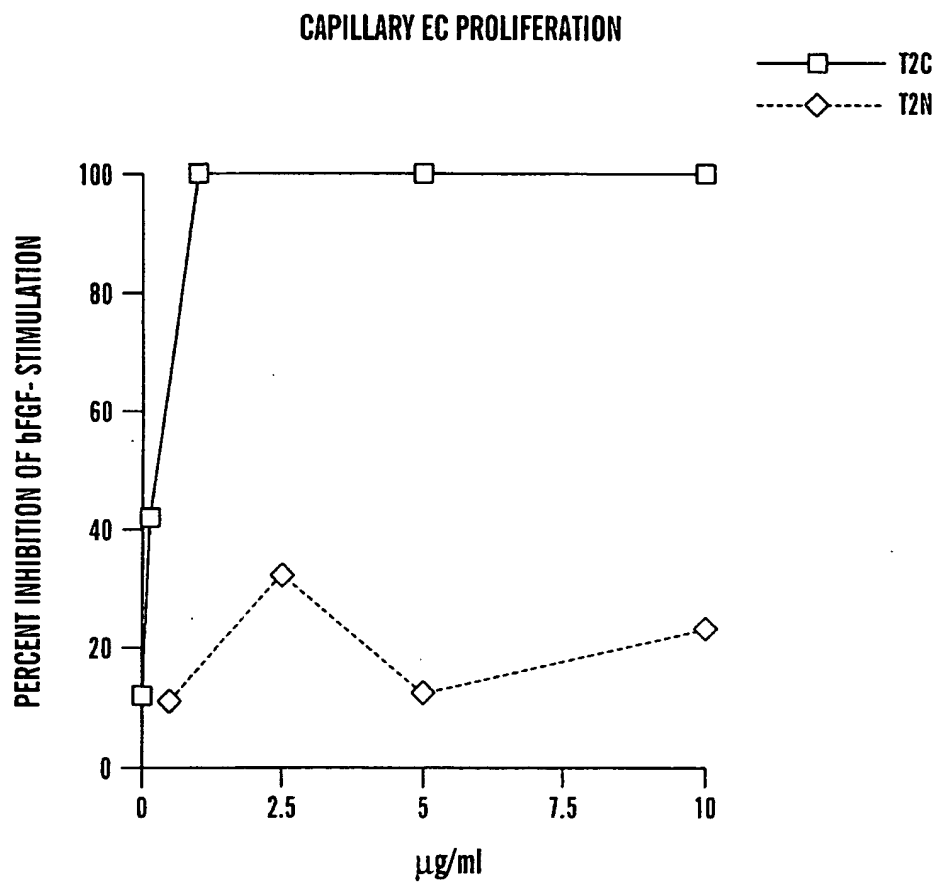
Accordingly, groups I-V are not so linked as by the same corresponding technical feature as to form a single general inventive concept.

**Continuation of B. FIELDS SEARCHED Item 3:** WEST 2.0, Medline, STIC: sequence search  
SEQ ID 1-7, antiangiogenic polypeptide, angiogenesis inhibitor, TIMP, TIMP 1-4, metalloproteinase inhibitor

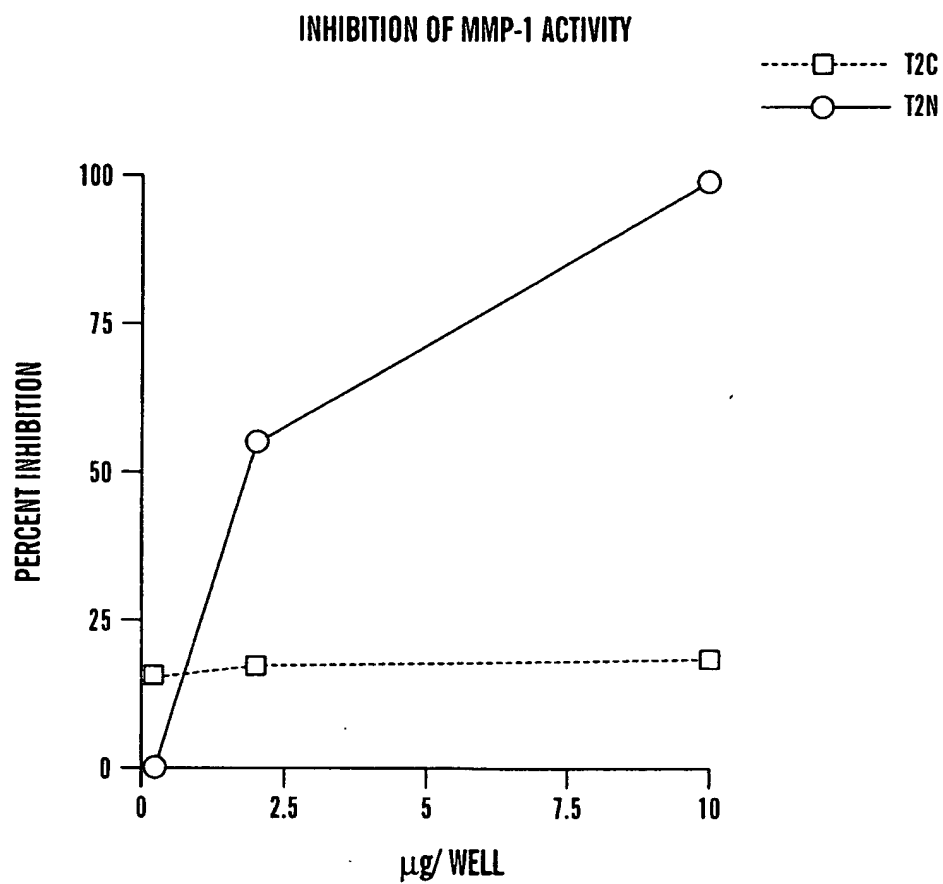
-24-

9. The method of claim 8, in which the tumor is a tumor of the central nervous system.
10. The method according to claim 7, in which the disease disorder is an ophthalmologic disease or disorder.
11. An isolated polynucleotide encoding the antiangiogenic polypeptide of claims 1-3.
12. A vector comprising the polynucleotide of claim 11.
13. A host cell comprising the vector of claim 12.

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**FIG. 1**

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**FIG. 2**

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/07156

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 38/00

US CL : 530/300

According to International Patent Classification (IPC) or to both national classification and IPC:

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.2, 219; 514/2; 530/300, 324, 325, 326

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Continuation Sheet

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,698,671 A (STETLER-STEVENSON ET AL) 16 December 1997 (16.12.1997), whole document, especially claim 1 peptide (iii).	1-5
Y	US 5,595,885 A (STETLER-STEVENSON ET AL) 21 January 1997 (21.01.1997), see columns 14-16.	1-5
Y	TAKIGAWA et al. Induction of angiogenesis in chick yolk-sac membrane by polyamines and its inhibition by tissue inhibitors of metalloproteinases (TIMP and TIMP-2). Biochemical and Biophysical Research Communications. 28 September 1991, Vol. 171, No. 3, pages 1264-1271, see page 1268.	1-3
Y	VALENTE et al. TIMP-2 over-expression reduces invasion and angiogenesis and protects B16F10 Melanoma Cells From apoptosis.	1

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"A"

document member of the same patent family

Date of the actual completion of the international search

27 June 2000 (27.06.2000)

Date of mailing of the international search report

02 AUG 2000

Name and mailing address of the ISA/US

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/07156

### Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:  
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-5

Remark on Protest

☐  
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/07156

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING** This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-5, drawn to an antiangiogenic polypeptide, a pharmaceutical composition and a method of treating neurovascular disorder.

Group II, claim(s) 6, drawn to a method of treating tissue remodeling.

Group III, claim(s) 7-9, drawn to a method of treating atopic angiogenesis in a subject.

Group IV, claim(s) 10, drawn to a method of treating ophthalmologic disorder.

Group V, claim(s) 11-13, drawn to polynucleotide sequences, a vector and a host cell containing the vector.

The inventions listed as Groups I-V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature linking groups I-V appears to be that they relate to an antiangiogenic polypeptide.

However, Takigawa et al. (1990) teach that metalloproteinases are involved in angiogenesis and this can be inhibited by polypeptides which are termed tissue inhibitor metalloproteinases (TIMP). Therefore, the technical feature linking the inventions of groups I-V does not constitute a special technical feature as defined by PCT rule 13.2, as it does not define a contribution over the prior art.

The special technical feature of group I is considered to be the polypeptide and a method of treating neurovascular disorder.

The special technical feature of group II is considered to be a method of treating tissue remodeling.

The special technical feature of group III is considered to be a method of inhibiting atopic angiogenesis.

The special technical feature of group IV is considered to be treating ophthalmologic disorder.

The special technical feature of group V is considered to be a polynucleotide sequence.

Accordingly, groups I-V are not so linked as by the same corresponding technical feature as to form a single general inventive concept.

**Continuation of B. FIELDS SEARCHED Item 3:** WEST 2.0, Medline, STIC sequence search  
SEQ ID 1-7, antiangiogenic polypeptide, angiogenesis inhibitor, TIMP, TIMP 1-4, metalloproteinase inhibitor